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14. ABSTRACT Our proposal focused on developing a versatile and generic technology platform that allows direct evaluation of theroles played by specific genes (and their products) in the genesis, progression and maintenance of breast cancer.We have constructed genetically modified mice in which c-myc or E2f3 have been modified so as to incorporate areversible Tet or Lac regulatable switch. This will allow us to switch off (and on) individual target gene and determine what role that gene plays in mammary carcinoma. In effect, we can model the efficacy, specificity and side effects of a drug designed to target that gene's function, either alone or in combination with existing therapies. To date we have generated the targeting constructs for these mice, tested the switchability of the construct in vitro,identified correctly targeted ES cells and generated chimeric mice. Once the targeted mutant mouse lines areestablished we will combine them with established models of breast carcinogenesis, which will allow us to evaluatetheir separate and combined roles in driving breast cancer progression, in the maintenance of established tumors,and as therapeutic targets.

15. SUBJECT TERMS
Therapeutic target, mouse model, target validation, oncogenic pathway

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Table of Contents

INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Identifying the optimal molecular targets for effective and specific treatment of breast carcinoma is limited by our ignorance of which molecular pathways or network nodes are critical for the initiation, evolution and, above all, maintenance of breast cancers. Our overarching hypothesis is that although tumors appear genetically complex, they most probably are dependent upon a very much more limited repertoire of mutations for their maintenance. To test this hypothesis, we proposed to construct a novel type of mouse cancer model in which endogenous genes encoding critical signaling molecules are modified so that there expression can be toggled on and off at will by the action of ligand-dependent heterologous repressors. Using such heterologous repressor targeting (HRT), we will directly ascertain the requirement for such signaling molecules in normal breast epithelial development and maintenance and for mutant forms of such molecules in driving and maintaining breast cancers. Our initial focus within the BCRP proposal is on c-*myc* and *e2f3* genes, both of which encode pleiotropic transcription factors whose deregulated activities are causally implicated in breast (and other) cancers.

BODY:

Statement of work

The objectives of the proposal are to

1. Construct genetically modified mice in which the endogenous c-*myc* and *e2f3* genes are rendered susceptible to ectopic control, so allowing their reversible repression (and subsequent re-expression) at any stage of breast tumor development.
2. Use these mice to determine directly the requirement for c-Myc and/or E2F3 at various stages of tumor development in an H-Ras-driven mouse model of breast cancer and evaluate the therapeutic utility of c-Myc and/or E2F3 inhibition in the treatment of breast cancer.
3. Establish the HRT technology platform as a means to assess the requirement for c-Myc and/or E2F3 in any orthotopic mouse model of breast cancer.

Time lines and milestones

Task 1

Construct mice in which the endogenous c-*myc* gene is rendered switchable by the IPTG-dependent LacI repressor and establish this in the WAP-H-Ras mammary tumor background.

a. *Construct LacI repressor c-Myc knock-in construct and verify by sequencing (months 1-4)*
This task was completed, although technical difficulties delayed the completion of the task.

b. *Electroporate construct into ES cells (transgenic core). Develop screening protocols (southern and PCR) to identify correctly targeted ES cells (months 4-5).*
This task has been completed. Screening protocols were successfully developed and validated that allow for identification of correctly targeted ES cells. To facilitate this, a coding-neutral change in the 5' sequence of c-*myc* exon 3 has been introduced into the Myc targeting vectors that will allow for one-step discrimination between targeted and endogenous c-*myc*. Figure 1 (appendix) shows the Genomic sequence of Myc. The targeting vector spanned Exon1 to Intron 3 and included a Neo cassette for ES cell selection, and the TRE or LacR repressor elements. An external probe was developed to discriminate between the wild type (wt) sequence and the correctly targeted mutant sequence. Panels B and C of Figure 1 illustrate the expected wt and mutant band sizes for two different enzymatic digests Bgl I or Bgl II of ES cell genomic DNA.
Purified DNAs have been electroporated into ES cells and positive recombinant clones have been identified.

c. *Select and expand ES cells and identify ES cells with correctly targeted knock in element (months 5-7)*
This task has now been completed. ES cells with targeted c-*myc* have been identified and cloned. Appropriate recombination has been verified by the strategies outlined above. Figure 2 shows a confirmatory Southern blot of genomic DNA from 7 ES cell clones. Three of these clones (3F1, 1F10, 1F5, indicated by stars) were selected for microinjection into blastocysts.

d. *Verify IPTG-dependent repression of c-myc in ES cells in vitro using TaqMan analysis specific for the modified c-myc transcript (month 8).*
This task has been partially completed. Endogenous c-myc expression has been tested and appears normal in the absence of the Lac Repressor and IPTG administration (data not shown). Repression has not been tested.

e. *Microinject ES cells into blastocysts (UCSF transgenic core) and generate chimeric mice (months 9-11)*
Chimeric mice have been generated from all three ES cell lines. To date we have generated a total of 31 chimeric mice (15 females, 16 males) for the LacI-Myc line.

f. *Breed positive chimeric mice to obtain germ line transmission of the modified c-myc gene (months 12-15)*
We have mated 12 of the chimeric male mice with C57Black6 females and generated at least 10 litters. All pups from all litters have been black indicating that the mutant allele was not present in the germline. We are continuing to breed these and the remaining chimeric males. We have also requested re-injection of the ES cells into blastocysts to generate more chimeras.

g. *[parallel] Start cross with Zp3Cre mice to excise Neo cassette left in the modified c-myc gene (month 14)*
This step cannot proceed until germline transmission is obtained

h. *Cross LacI-c-Myc KI with beta-actin-(NLS)LacI (months 15-17)*
The β-actin-(NLS)-LacI mice have been obtained from Dr. Heidi Scrabble and are with in our barrier facility. However this step cannot proceed until germline transmission is obtained.

i. *Cross HRT LacI-cMyc X beta-actin-(NLS)LacI into WAP-H-Ras mice to generate the WAP-H-Ras breast cancer model with IPTG-switchable c-myc (HRT LacI-c-myc X Wap-H-Ras mice) (months 17-20)*
This step cannot proceed until germline transmission is obtained.

j. *[parallel] Begin process of backcrossing of chimeras into C57Black6 background (will need 6 generations, eventually)*
This step cannot proceed until germline transmission is obtained.

k. *Expand colony of HRT LacI-c-myc X Wap-H-Ras mice.*
This step cannot proceed until germline transmission is obtained.

Task 2

Construct mice in which the endogenous c-*myc* gene is rendered switchable by the Doxycycline-dependent-*tTS*^{Kid} repressor and establish this in the WAP-H-Ras mammary tumor background.

a. *Generate Tet repressor c-Myc knock in construct and verify by sequencing (months 1-4).*
This task has been completed. See figure 1 for design of construct.

b. *Electroporate construct into ES cells (UCSF transgenic core). Develop screening protocols* (southern and PCR) to identify correctly targeted ES cells (months 4-5).
This task has been completed. See figure 1 for probes used to verify insertion of targeting vector.

c. *Select and expand ES cells. Identify ES cells with correctly targeted knock in element (months* 5-7).
This task has been completed. Figure 3 shows a confirmatory Southern blot of genomic DNA from 2 ES cell clones 3C1 and 1G5.

d. Verify doxycycline-dependent repression of c-*myc* in ES cells *in vitro* by Taqman expression analysis (month 8)
To verify doxycycline dependent repression of endogenous c-Myc the positive ES cell clones were transfected with a plasmid, ptTs expressing the Tet repressor. These cells were treated with or without doxycycline for 72 hours and then harvested for Western blotting. The blots were probed with Myc and actin antibodies. As shown in Figure 4 the repression of Myc was released by the presence of doxycycline. The original repression of Myc is incomplete because the ES cells are heterozygous, harboring one wt and one targeted mutant allele.

e. Microinjection ES cells into blastocysts (transgenic core) and production of chimeras (months 9-11)

Positive ES cell clones 3C1 and 1G5 were microinjected into blastocysts. 4 chimeras (3 males and 1 female) have been generated.

f. Breed positive chimeric mice to obtain germ line transmission of the modified c-*myc* gene (months 12-15)
Chimeric male mice have been bred with C57black6 females but to date germ line transmission has not been achieved. Breeding will continue and re-injection of blastocysts will be performed.

g. [parallel] Start cross with *Zp3Cre* mice to excise Neo cassette left in the modified c-*myc* gene (month 14)
This step cannot proceed until germline transmission is obtained.

h. Cross *HRT-tet-c-cMyc X beta-actin-tTS^{Kid}* mice (months 15-17)
This step cannot proceed until germline transmission is obtained.

i. Cross *HRT-tet-c-cMyc X beta-actin-tTS^{Kid}* into *WAP-H-Ras* mice to generate the *WAP-H-Ras* breast cancer model with Tet-switchable c-*myc* (*HRT tTS^{Kid}c-myc X WAP-H-Ras* mice*)* (months 17-20)
This step cannot proceed until germline transmission is obtained.

j. [parallel] Backcross of chimeras to C57Black6 (6 generations)
This step cannot proceed until germline transmission is obtained.

k. Expand colony of HRT *tTS^{Kid}c-myc X WAP-H-Ras* mice.
This step cannot proceed until germline transmission is obtained.

Task 3

Construct mice in which the endogenous *E2f3* gene is rendered switchable by the IPTG-dependent LacI repressor {or Tet repressor} and establish this in the WAP-H-Ras mammary tumor background.

a. *Generate LacI repressor E2F3 knock in construct and verify by sequencing (months 1-4)*
As previously reported the *e2f3* vector required more complex cloning strategies than initially considered. This was due to problems with obtaining the *e2f3* genomic sequence from the correct mouse strain but also because emerging evidence indicated that the *e2f3* gene encoded not one but two separate proteins, each with its own discrete set of properties. Most notably, *e2f3a* is a transcriptional activator that drives cells into cycle whereas the shorter *e2f3b* is a transcriptional repressor that damps down expression of the ARF tumor suppressor. In light of these unforeseen discoveries, the strategy was modified to generate vectors that would modify the endogenous *e2f3* gene to allow independent regulation of *e2f3a* and *e2f3b*. We have inserted a Tet-regulatable sequence just upstream of the *e2f3a* transcriptional start site that should, in principle, shut down expression of both *e2f3a* and *e2f3b* transcripts. In addition, *loxP* sites have been inserted either side of *e2f3a* exon 1 while *flt* sites frame exon 1 of *e2f3b*, see figure 5.) Thus, we will be able selectively to permanently ablate expression of either *e2f3a* or *e2f3b*, and then superimpose on this real-time regulation of the remaining e2f3 transcript.
This task has been completed; the constructs have been made and verified

b. Electroporate construct into ES cells (UCSF transgenic core). Develop screening protocols (southern and PCR) to identify correctly targeted ES cells (months 4-5).
This task has been completed, ES cells have been electroporated and screening strategies were developed.

c. Select and expand ES cells and identify ES cells with correctly targeted knock in element (month 5-7)
ES cells have been screened for insertion of mutant construct. 23 positive clones were identified for the tetE2F3 construct, 8 were expanded and verified. Two positive clones were identified for the tetE2F3-FL construct; both were expanded and verified.

d. Validate repression of E2F3 in ES cells *in vitro* by Taqman expression analysis (month 8)
Validation of repression was performed in vivo (see below) development.

e. Microinject ES cells into blastocysts (transgenic core) and production of chimeras (months 9-11).
This task has been completed. Three positive ES cell clones were picked for the tetE2F3 construct and used for microinjection into blastocysts. Both of the

positive clones from the tetE2F3-FL construct were used for blastocysts injection and chimeras were obtained.

f. Breed positive chimeric mice to obtain germ line transmission of the modified *E2f3* gene (months 12-15)
One of the tetE2F3 lines gave germline transmission. This line has been used for all subsequent experiments. The tetE2F3-FL chimeras have so far failed to yield germline transmission. New chimers have been generated and are currently breeding.

g. [parallel] Start cross with *Zp3Cre* mice to excise Neo cassette left in the modified *E2f3* gene (month 14)
This step has not been completed.

h. Cross *tet-E2f3* KI with *tTSkid repressor strain* (months 15-17)
The tTSkid line has been obtained from the Coughlin lab. The tetE2F3 line has been designated $E2F3^{TRE.}$ Homozygous mice, $E2F3^{TRE/TRE}$, have been generated and bred with the tTSkid line. $E2F3^{TRE/TRE}$;tTSkid mice have recently been obtained.
Mouse Embryo Fibroblasts (MEFs) were harvested from $E2F3^{TRE/TRE}$ mice and transfected with both a tasked expression plasmids. As expected, in the absence of Doxycycline E2F3 was not detectable. However addition of doxycycline did not relieve repression in this system. It is possible that this is due heterochromatin formation during development. Experiments are underway to generate MEFs from $E2F3^{TRE/TRE}$;tTSkid mice given doxycycline during pregnancy to determine if repression of E2F3 expression can be reversed.

i. Cross *HRT LacI-E2f3 X beta-actin-(NLS)LacI* into *WAP-H-Ras* mice to generate the *WAP-H-Ras* breast cancer model with IPTG-switchable *E2f3* (*HRT LacI-E2f3 X Wap-H-Ras* mice) (months 17-20)
This step has not been completed.

j. [parallel] Begin process of backcrossing of chimeras into C57Black6 background (will need 6 generations, eventually)
This step is underway.

k. Expand colony of *HRT LacI-E2f3 X Wap-H-Ras* mice.
To be completed.

Task 4

Determine effect of c-Myc and E2F3-inactivation on normal mouse tissues and on development and maintenance of H-Ras-induced breast adenocarcinoma *in vivo*

This task cannot be attempted until germline expression is achieved and regulation of the mutant allele has been verified

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

- Successful design and construction of targeting vector, LacI-cMyc, to render endogenous c-*myc* gene regulatable via the IPTG-dependent Lac Repressor.
- Successful targeting of ES cells with *LacI-cMyc* targeting vector.
- Generation of LacI-cMyc chimeric mice.
- Successful establishment of colony of *beta-actin-(NLS)LacI* mice.
- Successful design, construction of Tet-cMyc targeting vector to render endogenous c-*myc* gene regulatable via the tetracycline sensitive tTS^{kid} -repressor.
- Successful targeting of ES cells with Tet-cMyc targeting vector.
- Validation of Tet regulation of targeted c-myc.
- Generation of Tet-cMyc chimeric mice.
- Successful construction and checking of modified targeting vectors, tet-E2F3 and tet-E2F3-FL, to render the endogenous E2F3 gene regulatable. Modifications required because of new discoveries concerning the nature of gene products encoded by the *e2f3* gene and their disparate biological functions.

- Successful targeting of ES cells with tet-E2F3 and tet-E2F3-FL targeting vectors.
- Generation of tet-E2F3 and tet-E2F3-FL chimeric mice.
- Germline transmission of tet-E2F3 allele
- Generation of homozygous tet-E2F3 mice, designated $E2F3^{TRE/TRE}$.
- Generation of MEFs from $E2F3^{TRE/TRE}$ mice.
- Validation of suppression of E2F3 in $E2F3^{TRE/TRE}$ MEFs.
- Establishment of colony of tTsKid repressor mice.
- Generation of $E2F3^{TRE/TRE}$;tTsKid mice.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

manuscripts, abstracts, presentations;
patents and licenses applied for and/or issued;
degrees obtained that are supported by this award;
development of cell lines, tissue or serum repositories;
infomatics such as databases and animal models, etc.;
funding applied for based on work supported by this award;
employment or research opportunities applied for and/or
received based on experience/training supported by this award.

LacI-cMyc targeted ES cells
Tet-cMyc targeted ES cells
tet-E2F3 targeted ES cells
tet-E2F3-FL targeted ES cells
$E2F3^{TRE/TRE}$ targeted mutant mice

CONCLUSIONS: Summarize the results to include the Importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report.

We have successfully generated the targeting vectors and targeted ES cells required. Generation of germline transmission from chimeric mice has been problematic and we are continuing work to resolve these issues. Our preliminary investigation of the Tet regulation of targeted E2F3 allele confirmed the expected repression, however this repression was not relieved by the addition of doxycycline. The reason for the failure to de-repress is not clear but under investigation.

Due to the technical difficulties encountered with this project we have as yet been unable to test our hypothesis. Nevertheless we have continued other work to determine the therapeutic utility of inhibiting Myc in tumors and have demonstrated that inhibition of Myc transcriptional activity, using a dominant negative mutant of Myc called Omomyc, is sufficient to prevent or regress tumor formation in a K-*ras* lung tumor model, with out any significant or lasting side effects on normal tissue. We now intend to use this Omomyc technology in a breast tumor model.

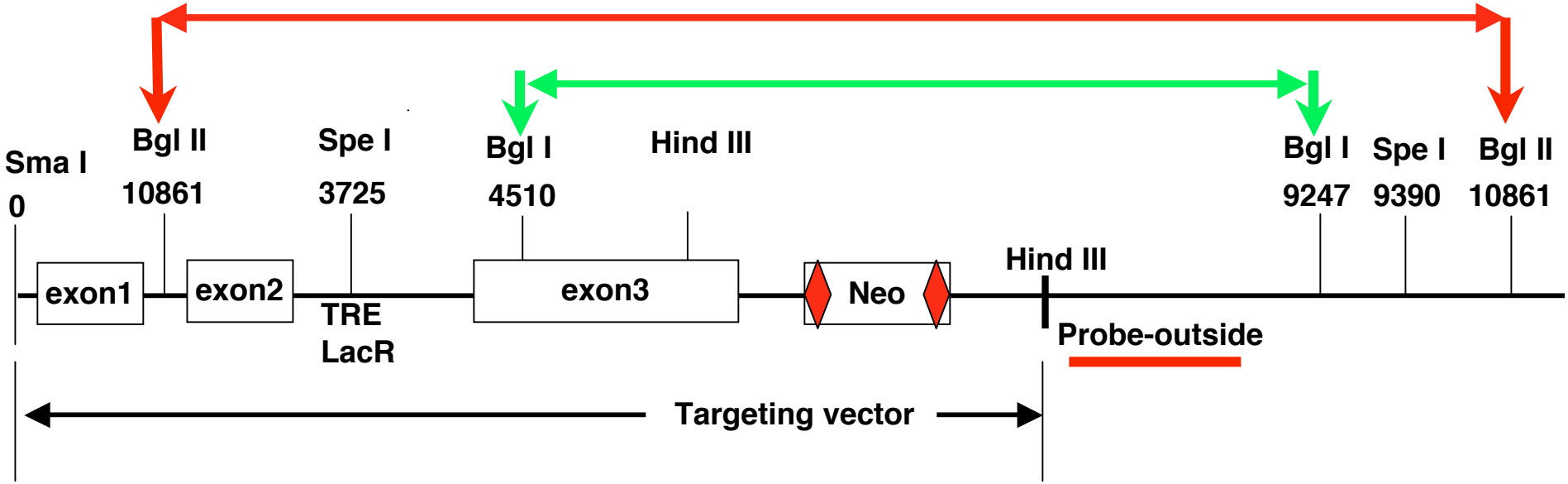
REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science, Military Medicine*, etc.).

APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

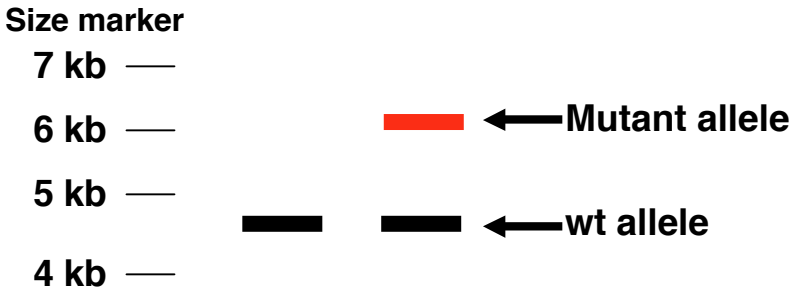
APPENDIX CONTENTS:

Figure 1.

A. **Schematic of c-Myc locus and targeting vector.**



B. Illustration of expected southern band sizes from Bgl I digest



C. Illustration of expected southern band sizes from Bgl II digest

Figure 2. Confirmatory Southern Hybridization of genomic DNA from ES cell targeted with *LacI-c-myc* vector.

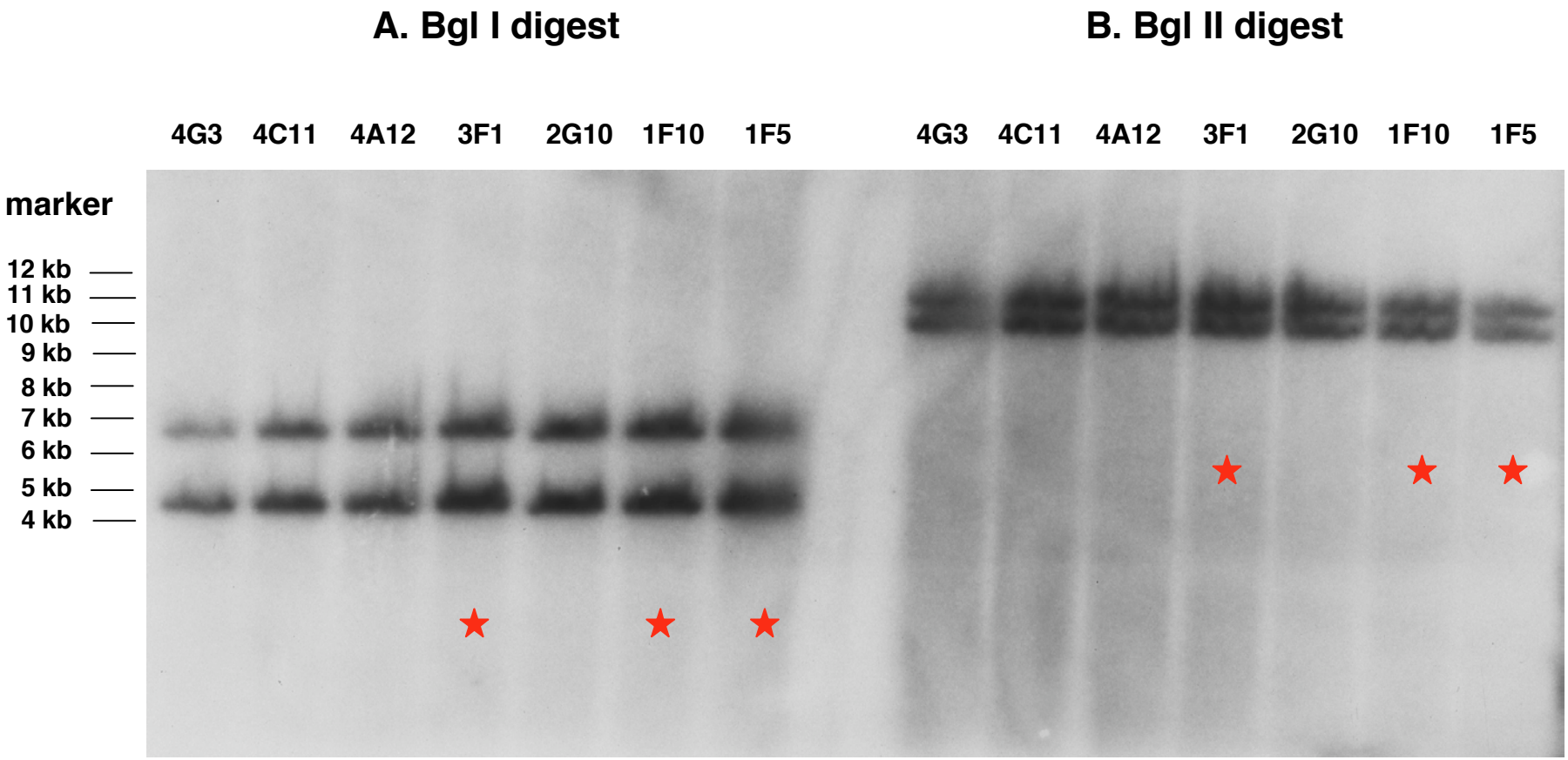


Figure 3. Confirmatory Southern Hybridization of genomic DNA from ES cell targeted with Tet-Myc vector.

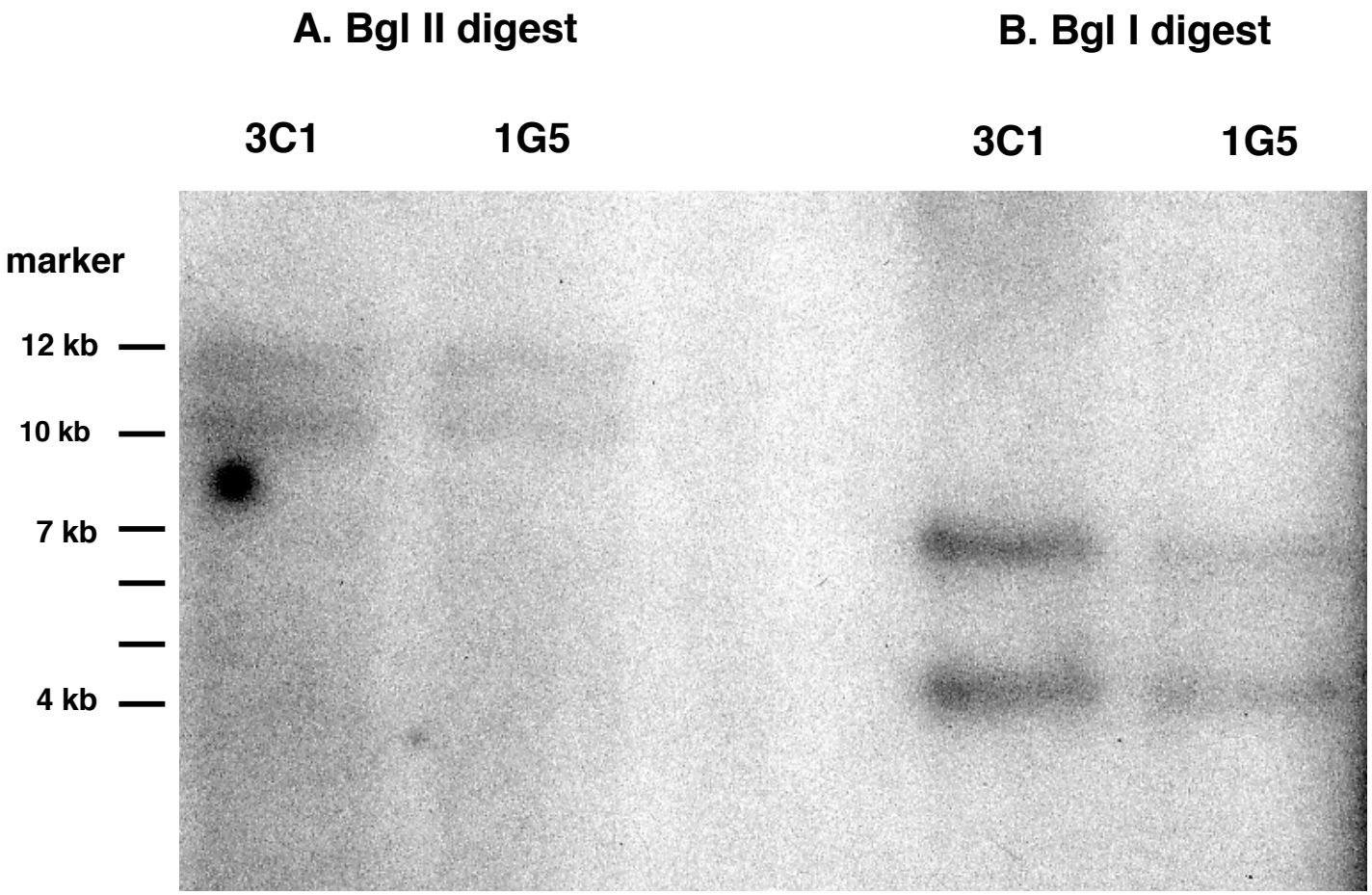
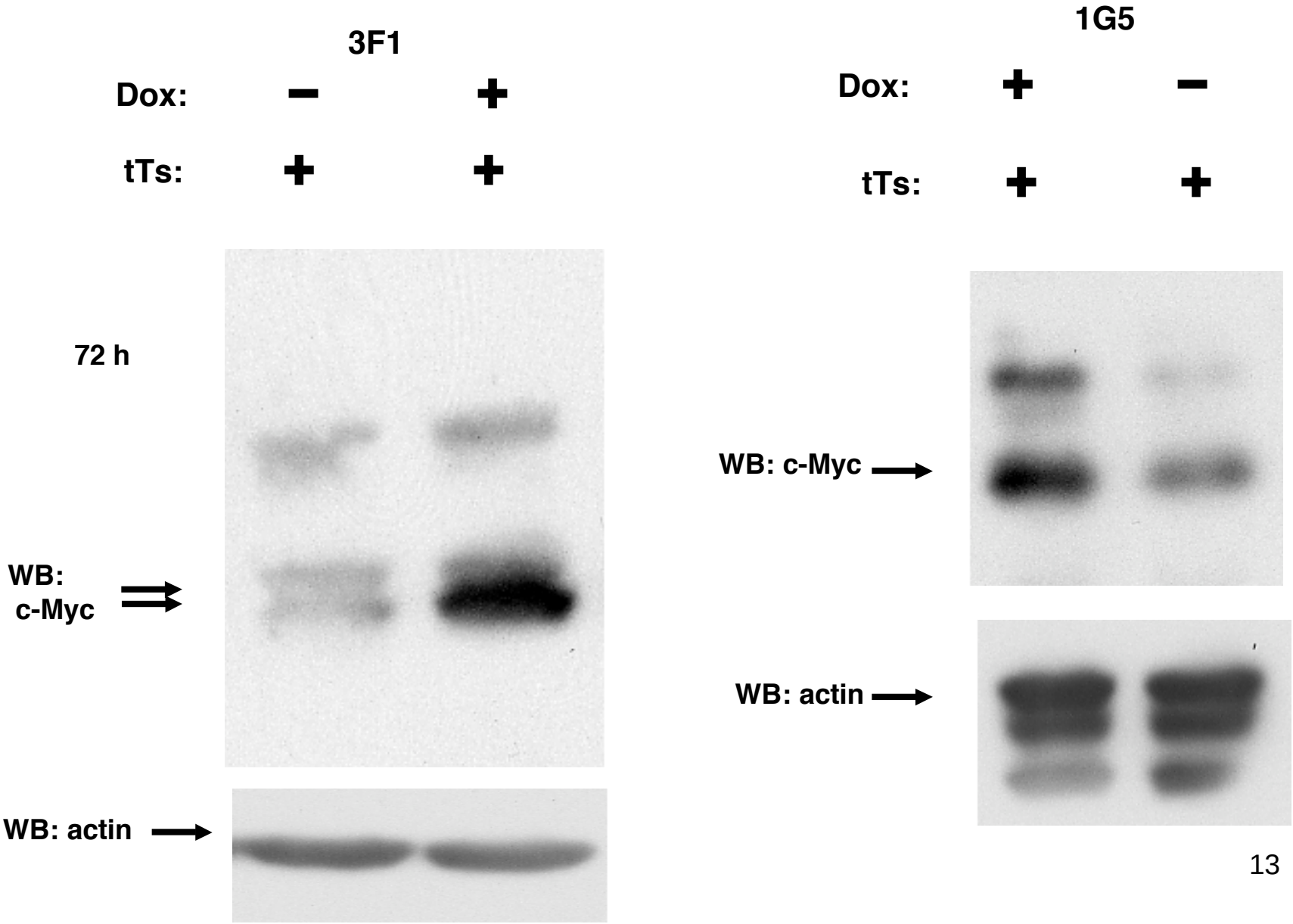


Figure 4. Validation of tet repression and doxycycline derepresion in targeted ES cells *in vitro*, transfected with ptTs



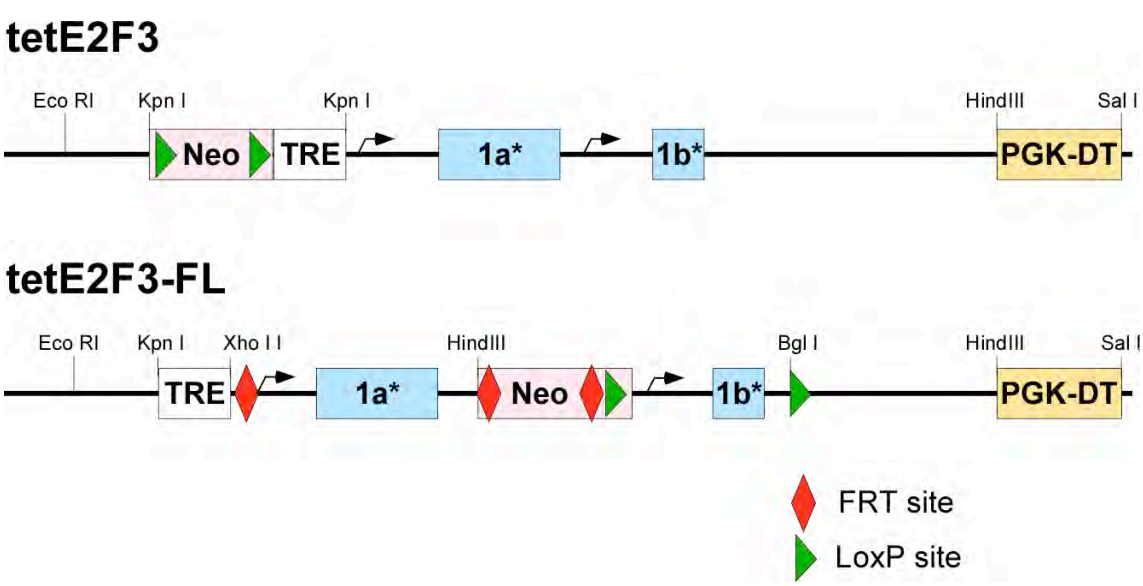
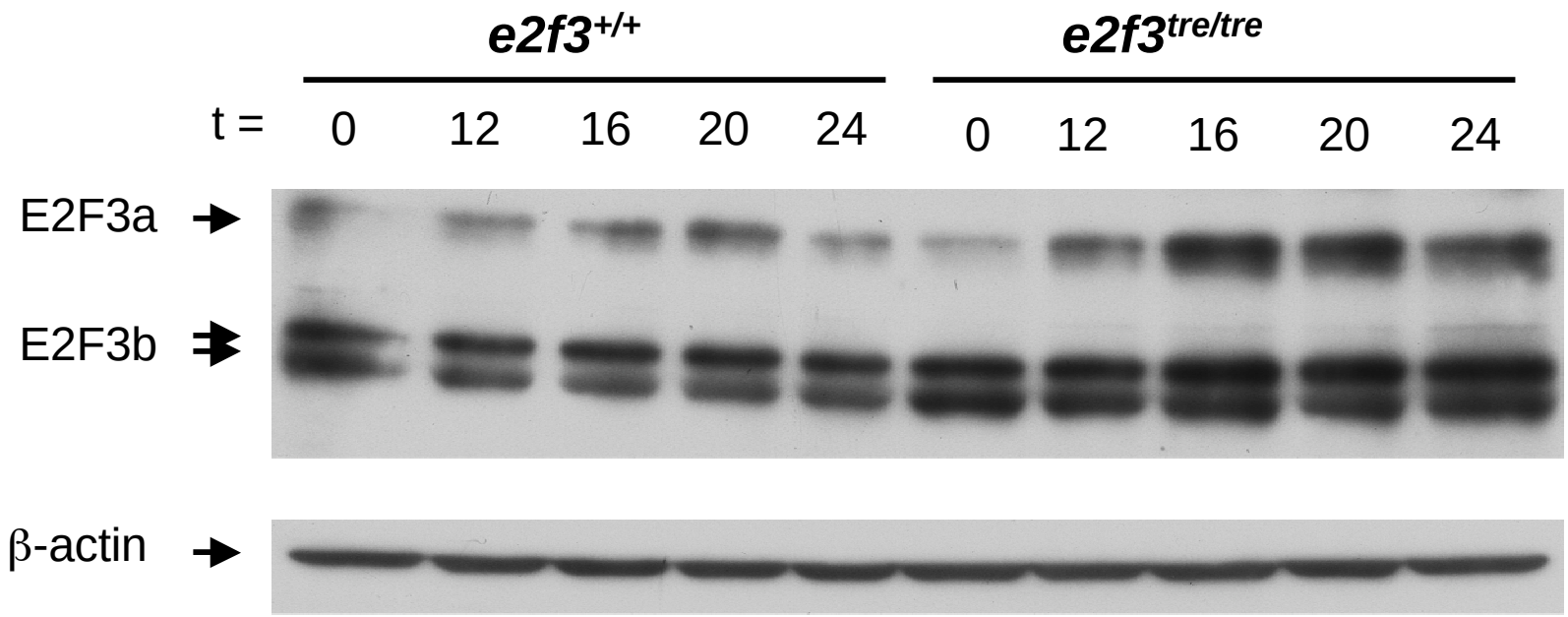


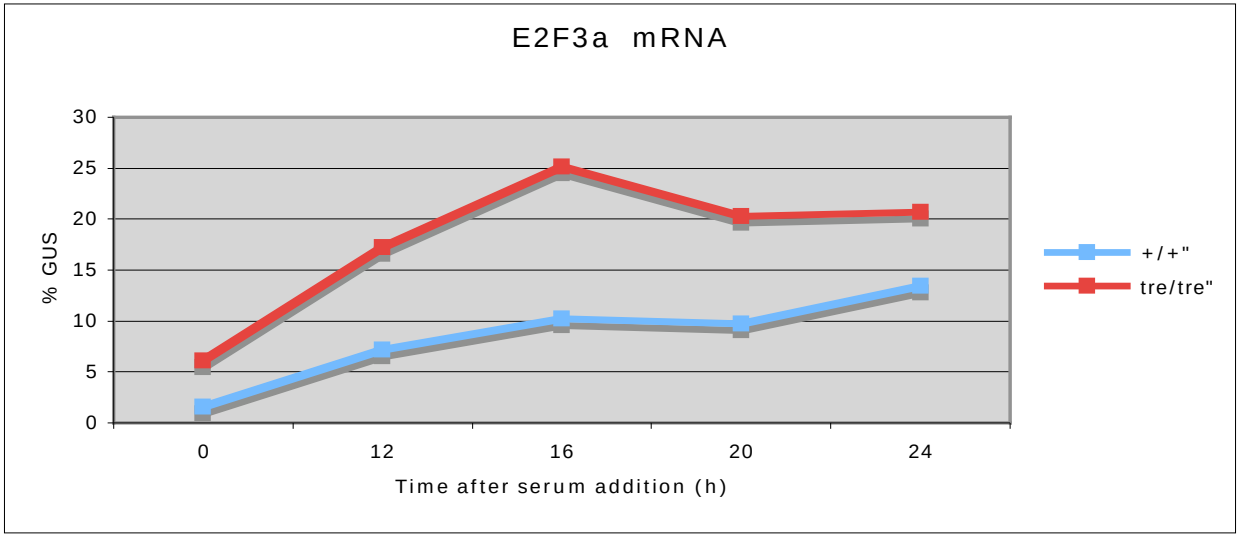
Figure 5
Diagrammatic representation of *e2f3* targeting vectors

Figure 6. E2F3 is expressed in knock-in homozygous MEFs, albeit at higher levels, and is regulated normally after serum stimulation. *e2f3tre/tre* MEFs show a normal cell cycle profile (data not shown).



Western Blot. Wild type and knock-in homozygous MEFs were serum starved for 48 hrs. Serum was added and protein extracts were prepared at the indicated times after serum addition.

Figure 7. Wild type and knock-in homozygous MEFs were serum starved for 48 hrs. Serum was added and RNA extracts were prepared at the indicated times after serum addition. cDNA was synthesized and E2F3 expression was measured by Taqman analysys.



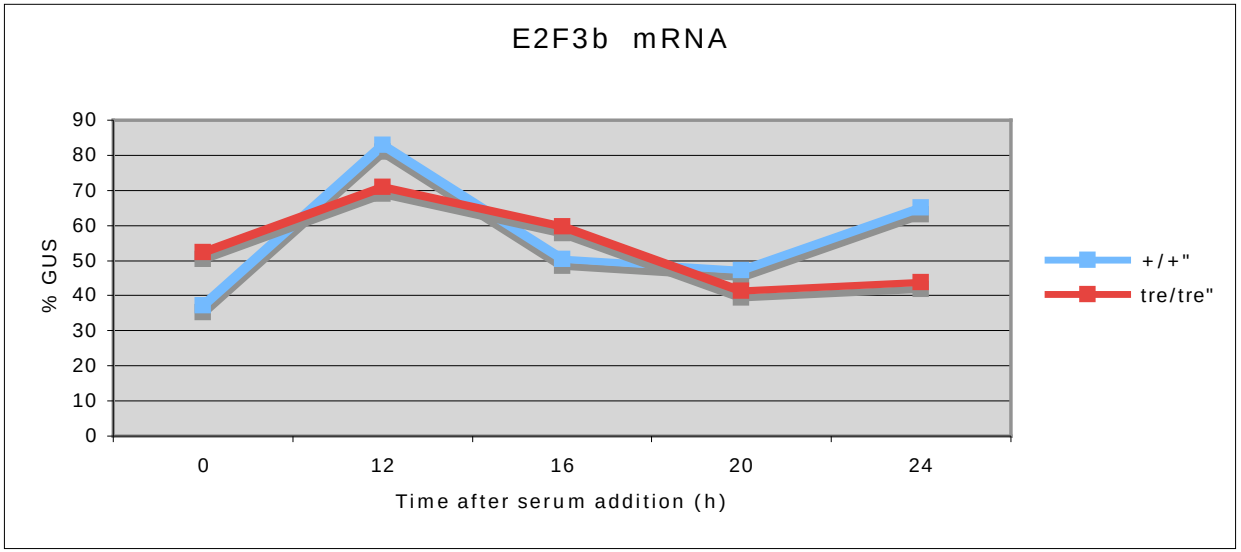
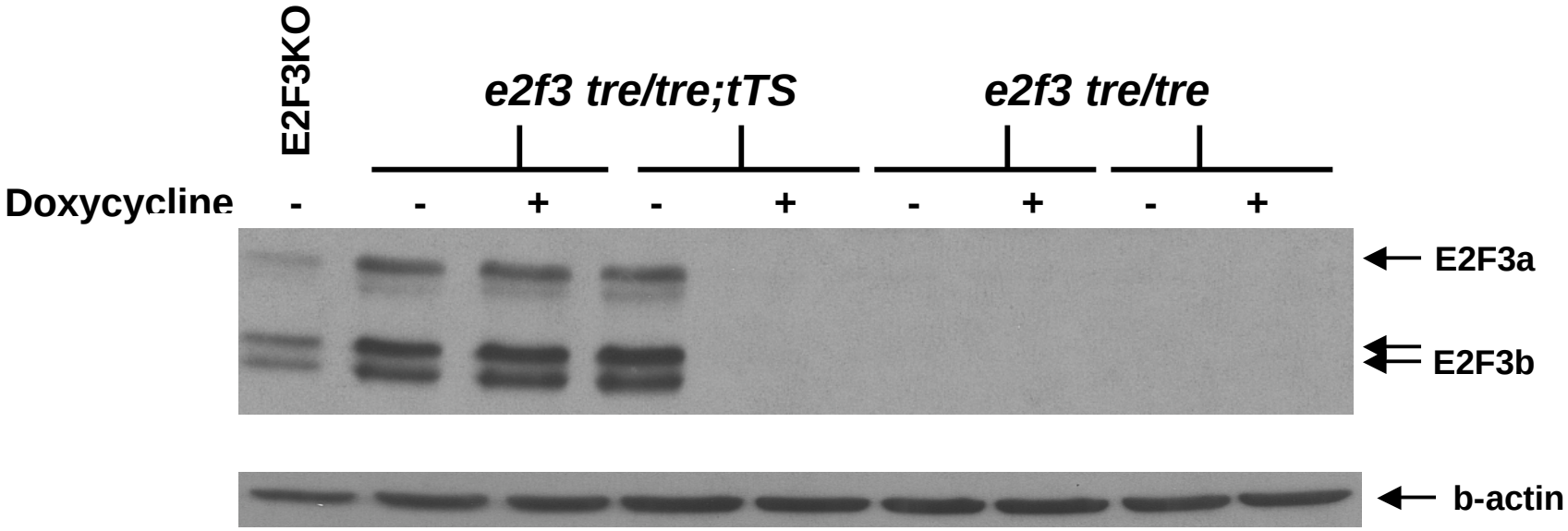


Figure 8: E2F3 is completely repressed in *e2f3tre/tre;tTS* MEFs extracted from embryos that developed without doxycycline. However, addition of doxycycline failed to release E2F3 repression in culture.



Western blot. MEFs with the indicated genotypes were extracted from embryos that developed without doxycycline. MEFs were plated, split in two at confluency and grown with or without doxycycline (as indicated) for 72 hours before protein extraction.

Same result by Taqman (not shown).